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Search for Localized Dysbiosis in Crohn's Disease Ulcerations by Temporal Temperature Gradient Gel Electrophoresis of 16S rRNA

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The mucosa-associated microbiota lining the gut epithelium might play a central role in the activation and/or perpetuation of mucosal inflammation in Crohn's disease (CD). We sought for localized dysbiosis by comparing the biodiversity and composition of the microbiotas in ulcerated and nonulcerated mucosal samples from patients with CD. Biopsy samples ($n = 75$) of ulcerated and adjacent nonulcerated mucosa were collected during colonoscopy from 15 patients, from the ileum, right colon, left colon, and rectum. Temporal temperature gradient gel electrophoresis (TTGE) of bacterial 16S rRNAs was used to evaluate the dominant bacterial species. TTGE profiles were compared using software that calculates similarity percentages. For a given patient, average similarity indexes between ulcerated and nonulcerated mucosal TTGE profiles ranged from 95.2% ± 4.2% to 97.9% ± 1.7% (means ± standard deviations) for the different segments. The mean values did not differ significantly. Average interindividual similarity indexes for a given segment among the different patients ranged from 33.6% ± 15.5% to 42.0% ± 25.6%. In CD, the dominant microbiotas do not differ qualitatively between ulcerated and nonulcerated mucosae. Biodiversity remains high in ulcerated mucosa. This argues against a pathogenic role of localized qualitative dysbiosis in CD-associated ulceration.

The intestinal microbiota is thought to have a role in Crohn's disease (CD) (18, 28). In susceptible individuals, CD onset may follow a breakdown of immunological tolerance to some endogenous microorganisms (6, 7) and/or be a result of dysbiosis (2). The intestinal ecology is difficult to study, largely because >50% of the bacteria present in the gastrointestinal tract cannot be cultured. The recent development of culture-independent techniques has led to major advances in this field (1, 5, 25, 26). Two strategies are commonly used to search for a role of microorganisms in inflammatory bowel disease (IBD), namely, the candidate microorganism strategy and the global description strategy. The former method has been used to study pathogens and some *Escherichia coli* strains (3, 4). It can be based on culture or on culture-independent methods using specific probes. Despite intensive research focusing on *Mycobacteria*, *Listeria*, and *Chlamydia*, an infectious origin of IBD has not been confirmed (2, 13, 20, 22, 30). The global description strategy uses probes for large groups of bacteria which constitute the "dominant microbiota." It does not provide information on microorganisms present at low densities but describes the dominant microbiota more extensively than any other technique.

Most ecological studies of CD have concerned the fecal microbiota and have provided evidence of dysbiosis. Several authors have reported that the fecal microbiota of patients with both active and inactive CD differs from that of healthy subjects. These differences include an increase in the fecal density of *Bacteroides vulgatus* and a decrease in lactobacilli and bifidobacteria (8). Elevated levels of *Enterobacteria* have also been reported for CD (11, 14, 24, 29). Using dot blot analysis and a molecular inventory method (a global description strategy), we have previously found large differences in the fecal microbiota among individuals with CD and the presence of species that are usually absent from the dominant biota of healthy subjects (17).

Bacteria interact with host cells along the mucous layer lining the gut epithelium. The mucosa-associated biota differs from the luminal biota (16, 32), and its dominant components are fairly constant along the colon. Owing to sampling difficulties, the mucosa-associated microbiota is poorly known, especially for inflammatory settings. It has been reported that the mucosa-associated microbiota is more abundant in IBD patients than in healthy controls (15, 23, 27).

The patchy nature of digestive tract ulceration in CD is unexplained. Postulating that local changes in the microbiota might favor ulceration, we used temporal temperature gradient gel electrophoresis (TTGE) to compare the qualitative compositions of the mucosa-associated microbiotas in ulcerated (U) and nonulcerated (NU) regions of the ilea and colons of CD patients. TTGE of 16S rRNA is a powerful technique for comparing the biodiversity of the dominant microbiotas in different biological samples. It is capable of separating bacterial sequences with the same size but different thermal stabilities (31). Since 16S rRNAs from different bacterial species have different nucleotide sequences in variable regions, their thermal stabilities are also different. This method gives profiles

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TABLE 1. Characteristics of patients with Crohn's disease at the time of sampling

Characteristic	Value
Gender (no. M/no. F)	6/9
Mean age (range)	37.6 (21–63)
CDAI[a] (Mean [range])	334 (160–460)
Disease location (no. of patients)	
Ileum	5
Ileocolon	7
Colon	3
Treatment (in previous mo) (no. of patients)	
Antibiotics	0
Sulfasalazine	0
Mesalazine	1
Steroids	4
Purine analogs	2
Methotrexate	2
Infliximab	0

[a] CDAI, Crohn's disease activity index.

TABLE 2. Distribution of biopsy samples

Patient no.	Biopsy in UM/Biopsy in NUM[a]			
	Ileum	Right colon	Left colon	Rectum
1	−/−	+/+	+/+	+/+
2	−/−	−/+	+/+	−/+
3	−/−	−/−	+/+	−/+
4	−/+	+/+	−/+	−/+
5	+/+	−/+	−/+	−/+
6	+/+	−/+	−/+	−/+
7	+/+	+/+	+/+	+/+
8	−/+	+/+	−/+	−/+
9	+/−	+/−	+/+	+/+
10	+/−	−/+	−/+	+/+
11	−/+	+/−	−/+	−/+
12	−/+	−/+	+/+	−/+
13	−/−	−/−	+/+	+/+
14	+/+	−/+	−/+	−/+
15	+/+	−/+	−/+	−/+

[a] +, biopsy taken; −, no sample available

corresponding to most of the dominant bacterial species present in complex microbial communities.

MATERIALS AND METHODS

Patients. Fifteen patients with active CD who had not received antibiotics within the previous 30 days were studied. Their characteristics are shown in Table 1. The protocol was approved by the local ethics committee, and informed consent was obtained from each patient before sampling.

Colonoscopy and biopsy. Colonoscopy was performed during CD flare-ups. The patients drank 4 liters of polyethylene glycol 4000 during the 12 h before colonoscopy in order to cleanse the colon. Colonoscopy was performed under general anesthesia with a videoendoscope. Biopsy was performed using sterile, single-use biopsy forceps. Samples (~0.5 mg) were taken from the ileum (I), right colon (RC), left colon (LC), and rectum (R). Ulcerated mucosa (UM) and adjacent nonulcerated mucosa (NUM) were sampled from each segment, if present. Samples were placed in Starstedt 2.2-ml screw-cap tubes, frozen immediately in liquid nitrogen, and stored at −80°C until analysis.

TTGE. (i) DNA isolation and rRNA gene amplification. Total DNAs were extracted from biopsy samples using the bead-beating method as previously described (24). To increase efficiency, nucleic acids were precipitated with isopropanol for 10 min at room temperature, followed by incubation for 15 min on ice and centrifugation for 30 min at 15,000 × *g* and 4°C. Pellets were resuspended in 112 μl of phosphate buffer and 12 μl of potassium acetate. After an RNase treatment and DNA precipitation, nucleic acids were recovered by centrifugation at 15,000 × *g* and 4°C for 30 min. The DNA pellet was finally resuspended in 30 to 100 μl of Tris-EDTA buffer. The DNA concentration and integrity were determined visually by electrophoresis on 1% agarose gels containing ethidium bromide. The primers GCclamp-U968 (5′ GCclamp-GAA CGC GAA GAA CCT TAC) and L1401 (5′ GCG TGT GTA CAA GAC CC) were used to amplify the V6 to V8 regions of bacterial 16S rRNA. PCRs were performed using HotStar *Taq* DNA polymerase (QIAGEN, Courtaboeuf, France) as previously described (24). Several dilutions of template DNA were tested if the presence of PCR inhibitors was suspected (1 and 3 μl of crude extract or 1 μl at a 10^{-1} dilution), and the highest PCR-positive dilutions were used for further analysis. PCR products were analyzed by electrophoresis on 1% agarose gels containing ethidium bromide to determine their size (433 bp) and approximate density.

(ii) TTGE analysis of PCR amplicons. We used the DCode Universal mutation detection system (Bio-Rad, Paris, France) for the sequence-specific separation of PCR products. Electrophoresis was performed as previously described (9, 24). Electrophoresis was run at 64 mA for 20 h at an initial temperature of 66°C with a ramp rate of 0.2°C/hour. Gels were stained in the dark by immersion for 30 min in a solution of SYBR green I nucleic acid gel stain (Roche Diagnostics, GmbH, Mannheim, Germany) and were read using a Storm device (Molecular Dynamics).

Calculations and comparisons. TTGE profiles were analyzed with GelCompar software, version 2.0 (Applied Maths, Kortrijk, Belgium), which takes into account the number of bands, their positions on the gel, and their intensities. The software translates each TTGE profile into a densitometric curve, drawing a peak for each band (with the area under the peak being proportional to the band intensity). A threshold area value was used to remove small peaks from the densitometric curves, as these can result simply from excess DNA loading of the gel.

PCR amplification was considered successful when the TTGE profiles bore at least three bands. A marker consisting of a PCR amplicon mix of seven cloned rRNA genes from different bacterial species was used to normalize the profiles (24). The analysis included between-pattern comparisons based on the Pearson coefficient, calculated as a measure of the degree of similarity. Similarity indexes (Pearson correlation method) were calculated for each pair of profiles. The analysis of TTGE patterns with GelCompar II software yields a spatial representation (dendrogram) based on the matrix of Pearson correlation coefficients and application of the unweighted-pair group method using arithmetic averages (UPGMA). The positive similarity threshold when comparing TTGE profiles for biopsy samples was previously defined as 92% (16). The dendrogram reveals clusters of microbiota components sharing high degrees of similarity. The threshold defining a cluster was set at 80%. Means were compared using paired Student's *t* test when the variances were equal, and otherwise using Wilcoxon's test. For each patient, we first compared the mean similarity indexes of microbiotas associated with UM and NUM from the same segment. We then assessed the dominant mucosa-associated microbiota along the distal digestive tract by comparing the mean similarity indexes for the NUM samples of the different segments. Finally, we studied the similarity indexes between the microbiotas associated with UM from the different segments of the digestive tract.

RESULTS

Colonoscopic findings. Colonoscopy with ileal inspection was performed in 11 cases. The endoscope did not reach the ileum in the other four patients because of stenosis of the ileocecal valve or colon (two cases each). At least one segment with ulcerated mucosa was observed in all patients.

Biopsy samples and PCR results. A total of 75 biopsy samples were collected (Table 2). PCR amplification of the V6-V8 regions of 16S rRNA was available for 70 samples. Two specimens of UM (RC patient 1 and RC patient 7) and three specimens of NUM (RC patient 1, R patient 5, and I patient 7) yielded fewer than three TTGE bands and were thus excluded from analysis.

Comparative analysis of TTGE profiles. Biodiversity, as assessed by the number of TTGE bands, was high in both UM and NUM. The mean numbers of bands were 11.5 (range, 3 to 20) and 12.3 (range, 6 to 19) for NUM and UM, respectively.

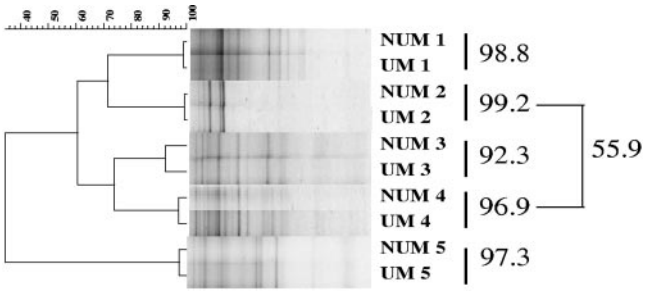

FIG. 1. TTGE of 16S rRNA gene amplicons (obtained using primers for the V6-V8 region) amplified from biopsy samples of ulcerated mucosa (UM) and nonulcerated mucosa (NUM) from the left colons of five CD patients. UM *X*, TTGE profile for UM of patient *X*; NUM *X*, TTGE profile for NUM of patient *X*. (Center) TTGE profiles ordered by Gel Compar II software. UM and NUM TTGE profiles for a given patient were always more similar than UM and NUM profiles for different patients. (Right) Pearson correlation coefficients yielded the calculated similarity indexes (expressed as percentages) of paired samples. (Left) The dendrogram represents a statistically optimal representation of the similarities between TTGE profiles based on the matrix of Pearson correlation coefficients and applying UPGMA.

A comparison of the numbers of bands for NUM and UM for a given segment did not show significant differences.

Intraindividual analysis. The TTGE patterns of five representative patients are shown in Fig. 1. The UM/NUM bacterial profiles of individual patients were always more similar (from 92.3% to 99.2% in the example) than the UM/NUM profiles of two different patients (55.9% in the example).

For given patients, ileal UM and NUM TTGE profile similarities ranged from 96.4% to 99.3%. The mean intraindividual similarity index between ileal UM and NUM TTGE profiles was 97.8% ± 1.2%. The mean similarity indexes between microbiotas associated with UM and NUM of the other segments were also very high, i.e., 97.9% for the right colon, 96.0% for the left colon, and 95.2% for the rectum (Table 3).

The mean similarity percentages between NUM sampled from the different stages of the intestine varied from 92.5% between ileal and left colon NUM to 95.2% between ileal NUM and NUM of the right colon (Table 4).

Moreover, a comparison of the microbiotas associated with UM in different sampling sites gave similar results. Similarity percentages ranged from 92.0% (right colon UM versus rectal UM) to 99.4% (ileal UM versus right colon UM).

No significant differences were found, regardless of the site of sampling, for either NUM or UM.

Interindividual analysis. Mean similarity indexes of TTGE profiles of samples collected from a given segment from different patients ranged from 33.6% ± 15.5% (ileum) to 42.0% ± 25.6% (rectum) for UM and from 37.7% ± 23.0% (ileum) to 43.2% ± 19.8% (right colon) for NUM (Table 5). The mean UM and NUM indexes did not differ significantly from each other for each location ($P > 0.05$). The mucosa-associated microbiota differed markedly from one patient to another. However, these differences were comparable for ulcerated and nonulcerated mucosae and were within the range of interindividual variability. Indeed, when all the TTGE profiles were compared in a single dendrogram, 15 clusters were obtained, corresponding to the 15 patients (Fig. 2). This indicated that the mucosa-associated microbiota of a given patient was stable from the ileum to the rectum and that it differed from one patient to another. There were no other clusters, showing that no specific dominant microbiota was associated with either ulceration or the site of disease involvement.

TABLE 3. Intraindividual similarity index between UM and NUM profiles for each segment of the distal intestinal tract

Tissue (*n*)	Similarity index (mean % ± SD) between UM and NUM
Ileum (8)	97.8 ± 1.2
Right colon (4)	97.9 ± 1.7
Left colon (14)	96.0 ± 3.8
Rectum (8)	95.2 ± 4.2

TABLE 4. Intraindividual similarity indexes between UM and NUM profiles for different segments

Tissue	Similarity index (mean % ± SD) between profiles[a]			
	Ileum	Right colon	Left colon	Rectum
Ileum	100	**95.2 ± 3.6**	**92.5 ± 7.1**	**92.9 ± 7.9**
Right colon	*99.4*	100	**94.4 ± 5.8**	**94.2 ± 2.9**
Left colon	*96.5 ± 2.3*	*93.2 ± 6.4*	100	**93.1 ± 4.5**
Rectum	*94.5 ± 3.7*	*92.0 ± 6.3*	*92.1 ± 6.4*	100

[a] Bold values compare NUM profiles of different tissues; italic values compare UM profiles of different tissues.

DISCUSSION

TTGE offers a profile of the dominant bacterial species present in a sample but does not identify the species individually (16, 24, 32). It allows an overview of the modifications encountered by the dominant bacterial species profile of a sample in relation to different factors. In this study, the dominant microbiotas of CD patients did not differ qualitatively between ulcerated and nonulcerated mucosae. Biodiversity was preserved, and no particular dominant microbiota appeared to be associated with ulceration. This does not support a pathological role of qualitative dysbiosis in CD-associated ulceration. As we previously showed (16), the NUM microbiota was represented by the same dominant species from the ileum to the rectum. Moreover, the microbiota of the UM was similar at every site of the distal digestive tract.

The microbiota differs markedly between the mucosal layer and the intestinal lumen, in both healthy individuals and IBD

TABLE 5. Interindividual similarity indexes for UM and NUM in each segment

Profile (*n*)	Similarity index (mean % ± SD)
UM I (7)	33.6 ± 15.5
UM RC (4)	40.5 ± 30.6
UM LC (7)	34.6 ± 16.1
UM R (5)	42.0 ± 25.6
NUM I (8)	37.7 ± 23.0
NUM RC (10)	43.2 ± 19.8
NUM LC (15)	39.2 ± 18.0
NUM R (14)	42.2 ± 17.9

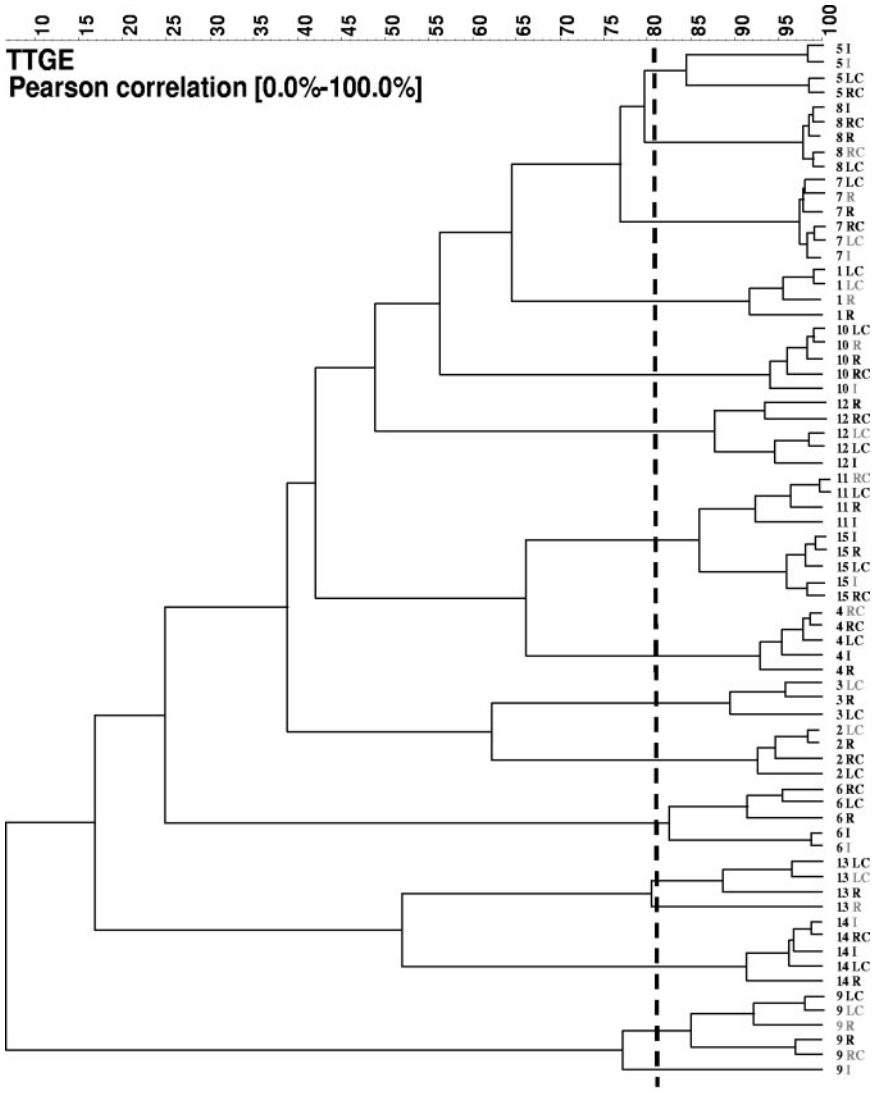

FIG. 2. Dendrogram representation of the TTGE profiles of 16S rRNA gene amplicons (obtained using primers for the V6-V8 region) amplified from biopsy samples of ulcerated mucosa (UM) and nonulcerated mucosa (NUM) from 15 CD patients. Biopsy samples were collected from the ileum (I), right colon (RC), left colon (LF), and rectum (R) of each patient. Gray designations, ulcerated mucosa; black designations, nonulcerated mucosa. The dendrogram represents a statistically optimal representation of the similarities between TTGE profiles based on the matrix of Pearson correlation coefficients and applying UPGMA. The vertical dotted line represents the threshold defining a cluster (80%).

patients (16). Some previous studies have examined mucosa-adherent bacteria after extensive washing and vortexing of biopsy specimens (27), but we preferred to study bacteria present in both the mucosa itself and its overlying mucus layer (23). Some authors have examined the mucosal microbiota by using both culture and culture-independent methods (15, 23, 27). Swidsinski et al. studied the microbiota of washed colonic biopsy specimens from patients with IBD and found that the bacterial density was higher in CD patients than in controls with ulcerative colitis, self-limiting colitis, or normal gastrointestinal status (27). The species composition was determined by culture and validated by quantitative PCR, cloning, and sequencing. No qualitative differences were found between IBD patients and healthy controls. Our results go beyond these data, as the dominant microbiota of ulcerated mucosa from CD patients did not differ qualitatively from that of the adjacent nonulcerated mucosa from the same patients.

No role of bacteria in the patchy nature of CD-associated ulceration has so far been established. In a study by Kleessen et al., fluorescent in situ hybridization with 14 16S/23S rRNA-targeted oligonucleotide probes was applied to surgical samples from 12 patients with ulcerative colitis, 12 patients with CD, and 14 non-IBD patients as controls (15). They observed differences in the species composition and in the extent of "bacterial penetration" between CD patients and controls. They did not specifically study ulcerated areas but reported that bacterial invasion was more pronounced in areas of erosion. Swidsinski et al. detected intracellular bacteria in patients with high densities of mucosal bacteria, whereas Schultsz et al., using fluorescent in situ hybridization with a single probe for

the *Bacteria* domain, found no intracellular bacteria in rectal biopsy specimens from IBD patients (15, 23, 27). In our study, no bacterial species was found to be specifically associated with CD ulceration, and ulceration did not qualitatively modify the dominant associated microbiota. Indeed, the microbiota associated with ileal ulceration or nonulcerated rectal mucosa in a CD patient was represented by the same dominant bacterial species.

It has been reported that the dominant luminal microbiota is unstable in CD patients, but we found that the dominant mucosa-associated microbiota was fairly consistent throughout the distal digestive tract of each given patient and that it did not differ between ulcerated and nonulcerated regions. This does not rule out a role of local dysbiosis in the pathogenesis of ulceration, as minority species may have a specific role (16, 24). One such candidate is a virulent pathovar of *Escherichia coli* described by Darfeuille-Michaud et al. (3, 4). Another candidate as an infectious cause of IBD is *Mycobacterium paratuberculosis*. Recently, Naser et al. (20) detected viable *M. paratuberculosis* in peripheral blood in a larger proportion of individuals with Crohn's disease than that of controls. Mylonaki et al. observed that *Bacteroides* and *Clostridium* spp. were more prominent and that *Bifidobacterium* and *Lactobacillus* spp. were less dominant in the rectal mucosa-associated microbiotas of patients with active IBD than in healthy controls (19). A high bacterial load itself could also induce tissue insult, either by facilitating bacterial penetration into the mucosa or by overstimulating the immune system with bacterial products such as muramyldipeptide, peptidoglycan, or lipopolysaccharide (10, 12, 21).

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